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Molecular-size standards for use in radiation-inactivation studies on proteins

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The accuracy of the radiation-inactivation technique for estimating molecular size was investigated with a range of proteins of known molecular mass. With the use of irradiation with a 16 MeV electron beam, inactivation was examined both in frozen samples at 77 K and in freeze-dried samples at room temperature. The effect of the presence of detergents and chloroplast membrane preparations was also measured. It was demonstrated that proteins added as internal standards, including malate dehydrogenase, glucose-6-phosphate dehydrogenase and cytochrome *c*, can provide an accurate calibration of molecular size. However, a disadvantage of the technique was that the target size of oligomeric enzymes could be that of either the monomers, dimers or higher oligomers. The detergent Triton X-100 increased the rate of inactivation of the proteins investigated.

INTRODUCTION

Radiation inactivation has been used to determine the functional molecular size (M_r) of both membrane-bound and soluble proteins [1]. The increasing doses of ionizing radiation applied to freeze-dried or frozen samples result in an exponential loss of biological activity. Analysis of the surviving activity uses the equation:

$$\log\left(\frac{A_D}{A_0}\right) = -KD$$

where A_D is the activity after dose D and A_0 is the initial activity at zero dose. K is a coefficient directly proportional to the M_r of the enzyme and represents the slope of a semi-logarithmic plot of surviving activity versus radiation dose. Target theory assumes that a single hit (primary ionization) on a macromolecule destroys its biological activity [1,2].

For many enzymes a linear relationship between the logarithm of surviving activity and radiation dose has been found. An empirical relationship between M_r and radiation dose was established by Kepner & Macey [1] for irradiation of freeze-dried samples at room temperature. This relationship, $M_r = 6.4 \times 10^5/D_{37}$ (where D_{37} is the radiation dose decreasing the original activity to 37%, i.e. by ln = 1), does not apply at lower temperatures, but either a correction factor [3,4] or a general equation:

$$\log M_r = 5.89 - \log D_{37} - 0.002t$$

(where t is the temperature in K) can be applied [5].

These empirical relationships are subject to errors due to sample preparation, irradiation dose and temperature. For greater accuracy it is necessary to use protein standards of known M_r (either mixed with the sample or externally).

Some recent studies have used usually one of a variety of M_r standards, acetylcholinesterase [6,7], malate dehydrogenase [8], glucose-6-phosphate dehydrogenase [9–12] and yeast alcohol dehydrogenase [13]. However, these have mostly been used to check the applicability of empirical formulae in a separate experiment and not for direct calculation of the unknown M_r.

Where standards have been used there has been disagreement about the suitability of particular enzymes. In the studies by Lo and colleagues [14,15] five enzymes were used as standards for irradiation of freeze-dried samples at room temperature. These included β-galactosidase, yeast alcohol dehydrogenase and pyruvate kinase, but in a subsequent study McIntyre & Churchill [11] found these three enzymes produced variable results in irradiation studies on frozen samples and recommended only glucose-6-phosphate dehydrogenase. An alternative is to use endogenous enzymes of known M_r [16]. However, suitable endogenous enzymes will not always be present.

The present study has investigated the accuracy of the radiation-inactivation technique for use in experiments on photosynthetic membrane–protein complexes. Several proteins of known molecular mass were irradiated under a variety of conditions, including in the presence of chloroplast membrane proteins. The results indicate that only certain proteins are suitable as M_r standards. These protein standards must be added to the sample containing the protein of unknown M_r to produce accurate results. This is unlike most previous studies, which relied only on the empirical formulae mentioned above. The present method gives a more accurate estimate of M_r than previous studies.

EXPERIMENTAL

Chloroplast and cyanobacterial membranes

Photosystem II from the cyanobacterium *Phormidium laminosum* was isolated with the use of the detergent lauryldimethylamine oxide [17,18]. The Photosystem II complex was suspended in buffer containing 10 mM-Hepes, 5 mM-sodium phosphate, 10 mM-$MgCl_2$ and 25% (v/v) glycerol, pH 7.5. Photosystem II from pea (*Pisum sativum* var. Feltham First) was prepared from chloroplasts with the use of the detergent Triton X-100 [19]. This Photosystem II was suspended in buffer containing 20 mM-Mes, 15 mM-NaCl, 5 mM-$MgCl_2$ and 20% (v/v) glycerol, pH 6.3. Photosystem I was prepared with the use of Triton X-100 [20] and resuspended in buffer containing 100 mM-Tricine, 200 mM-NaCl and 0.1% Triton X-100, pH 8.2. O_2 evolution was measured as in [13] for *P. laminosum* and as in [19] for pea Photosystem II.

Sample preparation

Either frozen or freeze-dried samples were prepared in order to minimize secondary reactions as discussed in [13].

(a) Frozen samples. Samples (0.3 ml), containing cryoprotectants and enzymes as specified, were flushed with N_2 for 30 min and placed in 0.3 cm-diameter silica tubes. Samples were then frozen and stored in liquid N_2 and transported packed in either liquid N_2 or solid CO_2 pellets.

(b) Freeze-dried samples. Samples (1 ml) containing enzymes as specified were frozen in 5 ml graduated Pyrex tubes and freeze-dried overnight. The tubes were then flushed with N_2, sealed with a glass stopper and stored at 4 °C until irradiated.

Irradiation procedure

Irradiation of frozen samples was carried out at 77 K [13] in liquid N_2 with a 16 MeV electron beam (MEL SL 75/2 instrument; Addenbrooke's Hospital, Cambridge).

Freeze-dried samples were irradiated under vacuum at room temperature (295–305 K) as in [14]. Tubes were cooled during irradiation by using a fan blowing air over a lead block cooled with solid CO_2.

Dose rate was 2 Mrad/min, and freeze-dried samples were irradiated in 4 Mrad treatments followed by a cooling period until the specified dose was achieved. Radiation dose was checked by using Perspex [poly-(methyl methacrylate)] dosimetry.

After treatment all tubes were flushed with N_2 before thawing or rehydration to remove O_2 and O_3.

Assays

All enzymes and reagents were purchased from Sigma Chemical Co. All enzymes were stable in solution at 0 °C, and to freezing and thawing except where stated. Enzymes were assayed at 20 °C or 25 °C with initial calibration of the control sample followed by duplicate or triplicate assays of each sample. Temperature variations during assays were limited to ± 0.5 °C. A Cary 219 spectrophotometer and 1 ml 1 cm quartz cuvettes were used.

Alcohol dehydrogenase

Yeast alcohol dehydrogenase (M_r 148000, four subunits) (EC 1.1.1.1) and horse liver alcohol dehydrogenase (M_r 80000, two subunits) were assayed in a reaction mixture containing 75 mM-sodium pyrophosphate, 10 mM-glycine, 75 mM-semicarbazide. 1 mM-EDTA, 1 mM-β-NAD^+ and 0.04% (v/v) ethanol, pH 8.9. Activity was measured by the production of NADH, monitored at 340 nm.

β-Galactosidase

Escherichia coli β-galactosidase (M_r 464000, four subunits) (EC 3.2.1.23) was assayed in a reaction mixture containing 100 mM-sodium phosphate, 10 mM-KCl, 1 mM-$MgSO_4$, 40 mM-2-mercaptoethanol and 2.5 mM-*o*-nitrophenyl β-D-galactoside, pH 7.0. Activity was measured at 420 nm as the production of *o*-nitrophenol.

Cytochrome *c*

Type III horse heart cytochrome *c* (M_r 12400) was measured as the height of the absorption peak (550 nm) in a spectrum taken from 580 to 480 nm. A sample oxidized with 0.24 mM-$K_3Fe(CN)_6$ was used as reference versus a sample reduced by a few grains of $Na_2S_2O_4$.

Lactate dehydrogenase

Pig muscle lactate dehydrogenase (M_r 146000, four subunits) (EC 1.1.1.27) was assayed in a reaction mixture containing 30 mM-Hepes, 50 mM-KCl, 0.5 mM-EDTA and 0.2 mg of bovine serum albumin/ml, pH 7.5 (buffer A); 0.2 mM-β-NADH and 2 mM-pyruvate were added, and activity was measured as the decrease in absorption at 340 nm.

Malate dehydrogenase

Pig heart cytoplasmic malate dehydrogenase (M_r 70000, two subunits) (EC 1.1.1.37) was assayed in buffer A with 0.1 mM-β-NADH and sample added before incubation. Activity was measured as the linear decrease in absorption at 340 nm after the addition of freshly made 1 mM-oxaloacetate.

Acetylcholinesterase

Human erythrocyte acetylcholinesterase (M_r 150000, two subunits) (EC 3.1.1.7) was assayed at pH 8.7 by a method using 5,5′-dithiobis-2-nitrobenzoate [6,21] and monitored as the increase in absorption at 412 nm.

Glucose-6-phosphate dehydrogenase

Enzyme from *Leuconostoc mesenteroides* (M_r 104000, two subunits) (EC 1.1.1.49) was assayed as described in [11].

Data analysis

This was performed by using a Minitab statistical program (Penn State University) for linear regression and analysis of variance. Enzyme activities were expressed as percentages of the control value, and target analyses were performed as described in [13]. Regression lines were checked for error by a plot of standard residuals versus dose. Molecular sizes of enzymes were compared directly by using the slopes of the inactivation regression lines. Standard deviations of slopes are given, and all slopes given are negative.

RESULTS AND DISCUSSION

Irradiation of frozen samples at 77 K

A linear inverse relationship between the logarithm of remaining enzymic activity versus radiation dose was seen for all of the enzymes investigated, indicating a single-hit single-target response. Molecular size was obtained from the rate of enzyme inactivation (R; Table 1), which was determined from the slopes of linear-regression plots. From the molecular size, the number of enzyme subunits can be determined by using the relative molecular masses given in the Experimental section. The M_r in Table 1 is the relative molecular mass of that number of subunits. The R/M_r ratios obtained were very similar for all the enzymes analysed (Table 1), indicating that rate of inactivation was a function of M_r. Slightly higher rates of inactivation were found for two of the enzymes, which had been irradiated in the presence of detergent (Table 1, Expts. A and B).

Erythrocyte acetylcholinesterase behaved as a monomer, confirming previous observations [6,7]. The target sizes obtained for the other enzymes confirm earlier reports for frozen samples [8,11] although freeze-dried β-galactosidase irradiated at room temperature was previously shown to behave as a tetramer [14]. The rate

Table 1. Radiation inactivation of enzymes at 77 K

Values are the means ± S.D. for two separate experiments except for liver alcohol dehydrogenase. All enzymes were irradiated as indicated in the Experimental section in buffer A containing 20% glycerol, except for Expt. A, where buffer A contained 25% glycerol and 0.05% Triton X-100, and Expt. B, where buffer A contained 25% glycerol and 0.05% lauryldimethylamine oxide. Rate of inactivation is the slope of the linear-regression line fitted to a semi-logarithmic plot of enzyme activity versus radiation dose (in Mrad). M_r values of proteins are given in the Experimental section. Abbreviation: DH, dehydrogenase.

Expt.	Protein	$10^3 \times$ Rates of inactivation R	$10^{-3} \times M_r$	No. of subunits	R/M_r
A	Acetylcholinesterase	9.3±0.5	75	1	1.24
B	Yeast alcohol DH	19.9±1.5	148	4	1.34
C	β-Galactosidase	13.2±0.9	116	1	1.14
D	Glucose-6-phosphate DH	11.5±0.9	104	2	1.11
E	Malate DH	7.2±0.6	70	2	1.03
F	Lactate DH	9.9±0.6	73	2–3	1.34
G	Liver alcohol DH	16.5±0.7	160	4	1.03
		8.4±0.5	80	2	1.05

of inactivation of lactate dehydrogenase indicated an M_r significantly larger than that of a dimer.

The difference in target size of liver alcohol dehydrogenase between similar experiments may have been caused by a failure to eliminate secondary reactions that increase the rate of inactivation [13]. It is unlikely that this would have only given a 2-fold difference, and so the exact cause is not clear. Liver alcohol dehydrogenase must be considered unreliable as an M_r standard.

The chemical processes causing inactivation are complex and have been discussed but not fully explained previously [2–5]. The variation between enzymes in the number of subunits contributing to the functional size can be explained by differences in the degree of transfer of ionizing energy to neighbouring subunits. This variation with different tetrameric enzymes giving targets equivalent to monomeric, dimeric or tetrameric structures (Table 1) is a disadvantage when the technique is applied to protein complexes of unknown size. Interpretation of target-size data will require knowledge of subunit sizes.

Irradiation of enzymes added to membranes

It was intended to use the M_r standards in experiments on photosynthetic membrane–protein complexes, and therefore the enzymes were also irradiated in the presence of these membranes. Acetylcholinesterase and β-galactosidase were unsuitable for use with photosynthetic membrane preparations owing to interference in their assay by pigment absorption. Liver and yeast alcohol dehydrogenases were both unstable when irradiated in the presence of Triton X-100 in the membrane preparations. However, malate dehydrogenase and glucose-6-phosphate dehydrogenase were reproducibly inactivated when irradiated under a variety of conditions.

Table 2 shows that the rate of inactivation of malate dehydrogenase in two preparations isolated with the use of Triton X-100 detergent (Table 2, Expts. B and C) was significantly higher than in other preparations. The presence of membranes (Table 2, Expt. D) alone or of a lauryldimethylamine oxide-solubilized Photosystem II (Table 2, Expt. A) did not significantly alter the rate of inactivation from that found without membranes or detergent (Table 2, Expt. E). This effect of Triton X-100 is also shown in Table 1, and has also been previously reported for membrane proteins [22]. After a literature survey the authors suggested using a correction factor. decreasing the M_r of all detergent-treated membrane proteins by 24%. It was suggested [22] that water-soluble proteins were unaffected. The results in Table 2 clearly show that a detergent effect on the apparent M_r of a soluble protein can occur and that this effect is not the same for all detergents. This confirms the need for the use of M_r standards, since use of empirical formulae even with correction factors would have given large errors in the calculated M_r. The cause of the Triton X-100 effect is not understood, but it is discussed by previous authors [22]. Therefore it is clear that the use of detergent-containing samples should be avoided where possible.

Glucose-6-phosphate dehydrogenase has been shown previously to be a good M_r marker (dimer M_r 104000, [11]), and this was confirmed (Fig. 1). Cytochrome *c* proved also to be a good marker where high radiation doses were required for low-M_r targets. The rate of inactivation indicated that the whole protein, including the covalently bound haem, behaved as a single target. A low-M_r standard can also be used to eliminate errors due to the gradually decreased solubility of samples found with increasing radiation. This decrease in

Table 2. Radiation inactivation of malate dehydrogenase

Malate dehydrogenase was added to each preparation, and irradiation was carried out at 77 K as indicated in Table 1. In Expts. B and C the sample contained Triton X-100. All values shown are the means ± S.D. for at least three separate experiments.

Expt.	Preparation	$10^3 \times$ Rate of inactivation
A	*P. laminosum* Photosystem II	7.4±0.3
B	Pea Photosystem II	8.8±0.4
C	Pea Photosystem I	8.9±0.3
D	*P. laminosum* membrane	7.7±0.5
E	Buffer A containing 20% glycerol	7.2±0.4

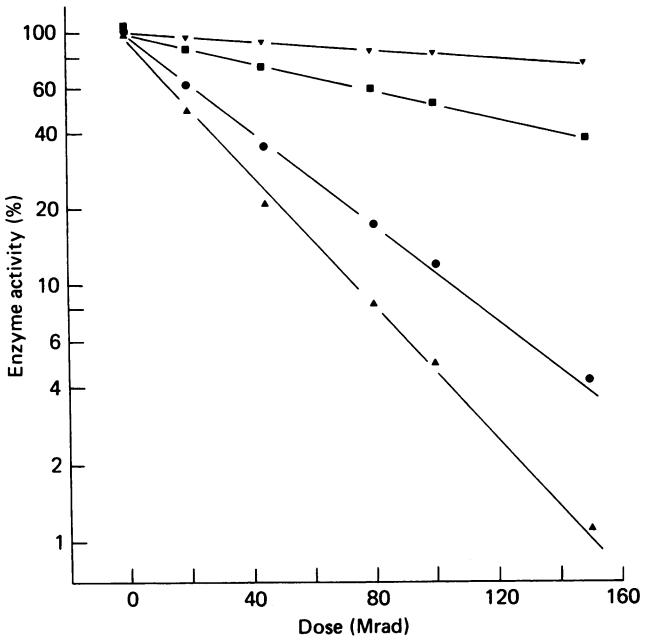

Fig. 1. Target-size analysis of glucose-6-phosphate dehydrogenase (▲), malate dehydrogenase (●), cytochrome *c* (■) and chlorophyll *a* (▼) added to a Triton X-100/Photosystem I sample

Frozen samples were irradiated at 77 K in 100 mM-Tris/HCl buffer, pH 8.2 containing 1 mg of bovine serum albumin/ml. Slopes of the regression lines (per Mrad) were: ▲, $1.35 \times 10^{-2} \pm 0.06 \times 10^{-2}$; ●, $9.1 \times 10^{-3} \pm 0.2 \times 10^{-3}$; ■, $2.7 \times 10^{-3} \pm 0.1 \times 10^{-3}$.

solubility is indicated by the chlorophyll *a* measurement, which gives an indication of sample concentration in Fig. 1. Cytochrome *c* can thus be used as a baseline (M_r 12500) and the slopes compared with this, giving a linear relationship between rate of inactivation and M_r.

Irradiation of freeze-dried samples

Irradiation of frozen samples at 77 K gave accurate results, but as shown in Fig. 1 very high doses of radiation are required for small proteins. At higher temperatures lower doses of radiation are required to achieve the same degree of inactivation. These temperature effects have been discussed previously [3,5] and show a linear inverse relationship between temperature and radiation dosage. This has previously been compensated for by either a correction factor [3] or an empirical equation involving irradiation temperature [5]. By using the same enzyme M_r standards as shown in Fig. 1, these correction factors were avoided. The rate of inactivation at room temperature was approx. 6-fold greater than that seen in similar samples frozen and irradiated at 77 K, but the relationship between radiation dose and M_r remained. The loss of solubility with increasing radiation dose was greater and more variable than seen in frozen samples, and correction of individual samples to a baseline of chlorophyll *a* (M_r 1000) or cytochrome *c* was required.

Conclusions

The present results show that the use of enzyme standards provides an accurate alternative to empirical formulae for M_r determination in radiation-inactivation experiments. A greater range of standards should be developed, and where possible endogenous enzymes present in the preparation under investigation should be used, as in [16]. Careful selection of standards and interpretation of results must be made. In the experiments presented here irradiation at 77 K produced more accurate and reproducible results than with samples irradiated at room temperature. The detergent Triton X-100 was confirmed to increase the rate of inactivation. It is suggested that where possible detergent preparations should be avoided, as there may be different detergent effects on soluble and membrane-bound proteins. The use of the technique on oligomeric enzyme systems has been shown to have limitations, but it will be useful to assign functions to subunits of already known size within complexes.

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